INVESTIGATION INTO ANTI-CANCER ACTIVITY OF D-LIMONENE IN CHRONIC MYELOID LEUKEMIA

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by

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A. Title of the thesis and abstract

Title:

Investigation into anti-cancer activity of D-Limonene in chronic myeloid leukemia

Abstract:

Chronic myeloid Leukemia (CML), a myeloproliferative malignancy of blood has attained importance nowadays because of its increased incidence. Treatment with tyrosine kinase inhibitors (TKIs) therapy has transformed the outcomes of patients with CML but resistance and associated adverse effects has lessened its efficacy. Natural products have been known for its enormous antioxidant and anticancer activities. D-Limonene, a monoterpene obtained from citrus fruits has been known for its potential chemopreventive and chemotherapeutic effects in various cancers. However, the underlying mechanism is still poorly understood. The aim of present study was to evaluate the effect of D-limonene in CML. In this study, *in vitro* effect of D-Limonene on K562 cells, a CML cell line was evaluated by MTT assay. D-Limonene has shown significant dose dependent reduction in % of cell viability of K562 cells (IC_{50}=3.6± 0.23 mM and 3.29± 0.19 mM at 24 and 48 h respectively) comparable with Doxorubicin. We have also evaluated the *in vitro* effect of D-Limonene on primary hepatocytes isolated from mouse. Treatment with D-Limonene did not show significant change on % of cell viability of primary hepatocytes. However, significant reduction in viability of primary hepatocytes was noted with Doxorubicin (IC_{50}= 14.13 µM) treatment. The *in vitro* antioxidant effect of D-Limonene (25, 50, 100, 200 and 400 µM) was evaluated by DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay in comparison with Trolox. D-Limonene has shown concentration dependent reduction in free radical formation in all assays except iron chelating assay. Further, the antioxidant activity of D-Limonene on K562 cells was evaluated by lipid peroxidation assay in which, D-Limonene has significantly reduced the production of malondialdehyde production in dose dependent manner. The *in vivo* effect of D-limonene (0.5, 1.0 and 1.5 g/kg, orally) was also determined on chemically immunocompromised K562 implanted xenograft C57BL/6 mice by subcutaneous and tail vein injection model for 14 and 17 days respectively. In subcutaneous injection model, D-Limonene treatment showed significant dose dependent reduction in tumor volume in K562 implanted xenograft mice as compared with disease control (DC) group. Furthermore, in tail vein injection model, treatment with D-Limonene has shown significant changes in haematological and biochemical parameters.

Significant reduction in WBC and NEU count was noted with D-Limonene treatment in all treatment groups as compared to the DC group. The change in LYM count, RBC count and Hb content was non-significant with D-Limonene at 0.5 g/kg; however at doses 1.0 and 1.5 g/kg, significant reduction in LYM count, whereas significant increase in RBC count and Hb content was observed as compared to the DC group. In biochemical parameters, significant dose dependent reduction in serum SGPT, SGOT, ALP, lipid peroxidation and nitrite production were observed in D-Limonene treated group as compared to DC group, whereas significant increase in superoxide dismutase level was observed with D-Limonene treatment as compared to DC group. We have also evaluated the effect of D-Limonene on angiogenesis by using CAM assay. D-Limonene (1, 5 and 10 µg/implant) has shown significant dose-dependent reduction in number of blood vessels on CAM as compared to implant soaked with the vehicle. Reduction in number of blood vessels on CAM was highest with D-Limonene at concentration 10 µg/implant. We have also evaluated the effect of D-Limonene on expression of VEGF protein by western blotting and MMP-2 and 9 proteins by gelatin zymography. D-Limonene (0, 1, 2, 3 and 4 mM) has shown significant dose dependent reduction in the expression of VEGF, MMP-2 and 9 proteins as compared to proteins from untreated cells. The inhibition of expression of VEGF, MMP-2 and 9 was highest at concentration of 4mM. In conclusion, D-Limonene has significantly inhibited the *in vitro* and *in vivo* growth of K562 cells without producing toxicity to normal hepatocytes. D-Limonene has presented appreciable antioxidant property in comparison to Trolox. It has also inhibited angiogenesis by inhibiting the expression of VEGF and invasion by inhibiting MMP-2 and 9, which makes it promising molecule for the treatment of CML.

B. Brief description on the state of the art of the research topic:

Malignancies of blood, affecting immature hematopoietic cells are termed as “leukemia”. It refers to both acute and chronic leukemias, depending on various morphological and clinical characteristics. Chronic myeloid leukemia (CML) is a type of myeloproliferative disorder characterized by an excessive formation of myeloid blood cells with presence of Philadelphia chromosome, formed by translocation at(9;22) that generates BCR-ABL fusion gene. This gene is responsible for generation of BCR-ABL oncoprotein that activates tyrosine kinase signaling (1; 2). The fusion protein lead to constitutive activation of tyrosine kinase receptor, forcing the cell to divide uncontrollably (3). According to WHO report in 2018, the global incidence of blood malignancy is 6% and CML accounts of about 20% of all leukemia affecting adults (4).

Researchers estimate nearly 0.3 million new cases and 0.23 million deaths will be projected with CML (5). Radiation exposure, age and gender are the common risk factor for CML (6). Chronic phase of CML is often asymptomatic at diagnosis and inexorably fatal if left untreated. The chronic phase progresses into an accelerated phase leading to a blast crisis in just 3–5 years, which gradually arrests the myeloid cell differentiation and accumulates immature blast cells in the circulation which eventually completely represses normal hematopoiesis, and vital functions of the body. Treatment with Tyrosine kinase inhibitors (TKIs) has shown profound benefit in survival and quality of life for CML patients by interfering with the tyrosine kinase receptors and signal transduction pathway of cell proliferation (7). Despite the significant efficacy and safety of TKIs, resistance, associated side effects and intolerance was noted in some of the patients. Monoterpenes, non-nutritive secondary metabolite found in the essential oils of citrus fruits and other plants have exhibited its anti-tumor activity in various types of cancers (8; 9). D-Limonene ((R)-1-methyl-4-(1methylethenyl) cyclohexene), a monocyclic monoterpene abundantly found in volatile oils of citrus fruits such as orange, lemon, grapefruit and others plants has inhibited neoplasia in animal models and cell growth *in-vitro* (10). Thus, it was hypothesized that D-Limonene may be a good anticancer agent for CML on virtue of its reported effect in other cancers. Therefore, the present study was aimed to evaluate its anticancer activity of D-Limonene in CML. For this study, we have selected K562 cell line, first human immortalized erythroleukemia type of myelogenous leukemia cell line. The cells were first derived from 53 year old female, from blastic phase. The K562 cells are non-adherent and round in shape with positive bcr:abl fusion gene. The cells also bear some proteomic resemblance to undifferentiated granulocytes and erythrocyte. Because of all these characteristics, K562 is the ideal cell line to study the CML (11).Further, various cell viability or cytotoxicity assays are available to check the effect of drug on cell line by *in vitro* method. MTT assay, the simplest colorimetric assay for assessing cell viability was selected to evaluate the cytotoxicity of D-Limonene on K562 cells. Drugs having cytotoxicity to cancerous cell may have chances to produce cytotoxicity to normal cells too. So, we have selected the primary hepatocytes from mouse as a source of normal cells and checked the cytotoxic effect of D-Limonene on it by MTT assay, compared to doxorubicin.

For *in vivo* study of CML, use of genetically modified mice was reported in literature but we have selected the C57BL/6 mice for our study. The particular strain and model was selected because of its availability, versatility, reduced mortality and cost-effectiveness. In this study, we

have developed two models of CML in C57BL/mice, for that immunosuppression was achieved by combination of Ketoconazole, cyclosporine and cyclophosphamide (12). The subcutaneous and tail vein injection model are ideal models for CML that helps to identify the therapeutic outcome, therefore both the models were selected for the study.

C. Definition of the Problem:

Current therapies for CML include allogeneic bone marrow stem cells transplantation and drug regimens. Stem cell transplantation is expensive, associated with substantial morbidity and mortality and is also limited to patients for whom a suitable donor is available. The drug regimens for CML includes interferon alfa, hydroxyurea and TKIs such as imatinib, nilotinib, dasatinib and ponatinib, among which imatinib and other TKIs are the treatment of choice in current chemotherapy of CML. TKIs induces marked responses in patients with CML and prolongs overall survival but the adverse effects and resistance are of much alarming nowadays (13; 14). It increases the occurrence of cardiovascular and pulmonary complications. It can alter the blood glucose regulation and also have chances to produce edema. Beside these adverse effects, mutations of BCR-ABL gene lead to loss of response or altered sensitivity of the TKIs. It indefinitely raises pharmacoeconomic burden, which is a cause of the concern globally and is also shifts the focus from efficacy to quality of life issues (15). Therefore, there is an emergence of appropriate treatment options for TKIs-resistant or -intolerant CML, that improves the efficacy of the treatment with fewer side effects.

D. Objective and Scope of work:

(A) Objectives:

1. To study the effect of D-Limonene on K562 (CML) cells and on primary hepatocytes by MTT assay.
2. To evaluate the *in vitro* antioxidant effect of D-Limonene.
3. To study the *in vivo* effect of D-Limonene in immunocompromised C57BL/6 mice by subcutaneous and tail vein injection model of CML.
4. To identify the possible mechanisms of D-Limonene involved in CML using,
    - Chick chorioallantoic membrane (CAM) assay of angiogenesis.
    - Expression of angiogenic factor VEGF by western blotting
    - Expression of tissue invasion and metastasis marker MMPs by gelatin zymography.

(B) Scope of work:

The Philadelphia chromosome or BCR-ABL fusion gene 'turns on' the tyrosine kinase signaling in myeloid cells that disturbs the downstream signaling pathways causing enhanced proliferation, differentiation arrest, and resistance to cell death. However, little is known about BCR/ABL-driven effector molecules contributing to the pathophysiology and clinical outcome of CML. Vascular endothelial growth factor (VEGF), one of the major angiogenic and capillary leak–inducing factor is expressed in a variety of human solid tumors and has been implicated in tumor-associated angiogenesis. Recent studies have reported that VEGF is also overexpressed in neoplastic myeloid cells and may play a potential role in human leukemias. In CML increased expression of VEGF in serum and affected bone marrow has been reported (16). Furthermore, BCR-ABL gene is also involved in regulation and expression of matrix metalloproteinases (MMPs). Expression of MMPs are not only concerned in normal processes but also in pathological processes, such as tumor invasion and metastasis (17; 18). In CML, stimulation of angiogenesis by angiogenic factors including VEGF and MMPs plays an important role in the pathogenesis of the disease. Apart from this, BCR-ABL is also involved in generation and accumulation of oxidative stress in CML. It was confirmed that BCR-ABL fusion protein is associated with augmented levels of ROS and tyrosine kinase activity in hematopoietic cells compared with their non-transformed parental counterparts (19). Therefore, there is a need of the treatment that can lower ROS through targeting directly the ROS-producing enzymes or would supplement the antioxidative potential of cells (20). Secondary metabolites from plants, such as alkaloid, flavonoids, glycosides, tannins, resins and volatile oil are gaining importance as a source of medicine for its lesser associated side effects in wide variety of human diseases including cancer (21). D-Limonene has been reported for its antioxidant, anti-inflammatory and gall-stone dissolving property (22-24). Beside this, D-limonene has well-established chemopreventive and chemotherapeutic activity against many types of cancers. It has inhibited neoplasia in *in vivo* animal models and *in-vitro* cell growth (10). It is found that oral administration of D-Limonene exhibited anticancer activity by inhibiting the growth of rodent pancreatic, mammary and gastric carcinogenesis (25-29). These observations suggest that it can be viewed as a conceptually promising agent in cancer therapy of CML.

E. Original contribution by the thesis:

D-Limonene has been reported as an anticancer agent in various types of cancers. However, it's *in vitro* and *in vivo* efficacy, safety, antioxidant activity and association with angiogenesis and expression of angiogenic factors have not been evaluated in any malignancy including CML. In this study, we have evaluated the effect of D-Limonene in CML and its progression. For this, the *in vitro* effect of D-Limonene was evaluated on proliferation of K562 cells and primary hepatocytes from mouse. Oxidative stress also plays a crucial role in disease progression. Therefore we have evaluated *in vitro* antioxidant activity of D-Limonene by DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay. We have evaluated the activity of D-Limonene on K562 cells by lipid peroxidation assay. Furthermore, the *in vivo* animal models for CML in immunocompromised C57BL/6 mice were developed by subcutaneous and tail vein injection of K562 cells and evaluated for effect of D-Limonene on disease progression. However, the mechanisms involved for its anticancer activity were still poorly understood from these studies. Therefore, the chick chorioallantoic membrane (CAM) assay was performed to study the effect of D-Limonene on angiogenesis on CAM surface. For the mechanistic study, we have evaluated the effect of D-Limonene on expression of VEGF and MMPs proteins by western blotting and gelatin zymography respectively. From all these assays, it is suggested that D-Limonene inhibits the *in vitro* and *in vivo* proliferation of K562 cells without any toxicity. Further D-Limonene has potential antioxidant and antiangiogenic properties; along with this it has inhibited the expression of VEGF and MMPs that may attribute to its anticancer activity in treatment of CML. Thus, this thesis will provide a scientific basis for further elaboration of complicated pathway involved in CML and effect of D-Limonene on its signaling.

F. Methodology of Research, Results / Comparisons:

Characteristics of cells

K562 is an erythroleukemia type CML cell line with spherical shape (Diameter-20 µ) and presence of BCR-ABL fusion gene. It bears some proteomic resemblance to undifferentiated granulocytes, monocytes and erythrocytes with mean doubling time of 12 h (30). Primary Hepatocytes isolated from mice is an adherent cell line, elongated and flat and in shape after attachment to the surface. Mean doubling time of 37 h (31).

Cell culture condition

K562 was procured from NCCS (National Centre for Cell Science, Pune, India) and maintained in RPMI-1640. Primary hepatocytes were isolated from mouse in our laboratory and maintained in DMEM medium. Both the culture media were supplemented with 10% FBS with 1 % antibiotic-antimycotic solution. Cells were maintained in an incubator at 37˚C supplemented with 5% CO_2.

1. Effect of D-Limonene on viability of K562 cells by MTT Assay

Effect of D-limonene on the viability of K562 cells was evaluated in time and dose dependent manner using 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay (32). K562 cells (10^4 cells/well) were treated with D-Limonene at concentration 1, 2, 4 and 8 mM and Doxorubicin as a standard at concentration of 0.4, 0.8, 1.6 and 3.2 µM for 24 h and 48 h.

D-Limonene at concentration of 1, 2, 4 and 8 mM shows significant reduction in % of cell viability of K562 cells as compared to untreated cells at 24 h (IC_{50}=3.6 ± 0.23 mM) and 48 h (IC_{50}=3.29 ± 0.19 mM) of treatment. However, no significant difference was observed in IC_{50} value at 24 h and 48 h of treatment. The reduction in % of cell viability of Doxorubicin was comparable with D-Limonene.

2. Isolation of primary hepatocytes from mouse and primary culture preparation

Mouse was anaesthetized and abdomen was opened to cannulate the portal vein with perfusion buffer (33). Gradually the perfusate flow was increased to wash out the blood from the liver (up to 20ml/min) making an incision in vena cava allowing perfusate efflux. The liver was blanched and was dissected out; perfusion was continued with warm collagenase (type II) buffer (0.05 %).The liver was transferred to the petri dish containing ice-cold suspension buffer containing calcium and magnesium). Gilsson's capsule was dissected and suspension was filtered through cell strainer. Debris were discarded and washed with wash buffer (perfusion buffer containing calcium) by centrifugation (50g for 5 min.) and resuspension (34; 35).

Cell viability was determined using trypan blue method and cells were cultured in DMEM containing 10% FBS and maintained in an incubator at 37˚C supplemented with 5% CO_2 atmosphere.

3. Effect of D-Limonene on cell viability of primary mouse hepatocyte by MTT assay

Treatment with Doxorubicin and D-Limonene was given to primary hepatocytes isolated from mouse at different concentration followed by 24 h of plating. Cells were treated with Doxorubicin at concentration of 2, 4, 8, 16 and 32 µM and D-Limonene with concentration of 2, 4, 8, 16 and 32 mM for 48 h. The cytotoxicity of D-Limonene and Doxorubicin on primary hepatocytes was compared by MTT assay.

Treatment with D-Limonene did not show significant reduction in % cell viability of normal mouse primary hepatocytes compared to untreated cells by MTT assay. However, Doxorubicin significantly reduced the % of viability of mouse primary hepatocytes as compared to untreated cells (IC_{50}= 14.13 µM).

4. Estimation of *in vitro* antioxidant activity

The *in vitro* antioxidant activity of D-Limonene was determined by DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay in comparison with Trolox as a standard (36; 37).

4.1. Test solution preparation

Test solutions of D-Limonene at concentration of 25, 50, 100, 200 and 400 µM and Trolox at concentration of 10, 25, 50, 100 and 250 µM were prepared.

4.2. Effect of D-Limonene on DPPH assay (37)

DPPH assay of D-Limonene (IC_{50}= 384.73 µM) has shown appreciable concentration dependent reduction in free radical formation in comparison with Trolox (IC_{50}= 153.30 µM) as a standard.

4.3. Effect of D-Limonene on ABTS assay (37)

D-Limonene (IC_{50}= 603.23 µM) has shown appreciable concentration dependent reduction in free radical formation by ABTS assay in comparison with Trolox (IC_{50}= 203.37 µM) as a standard.

4.4. Effect of D-Limonene on FRAP assay(37; 38)

D-Limonene (IC_{50}= -589.85 µM) has showed appreciable concentration dependent reduction in free radical formation by FRAP assay in comparison with Trolox (IC_{50}= -171.73 µM) as a standard.

4.5. Effect of D-Limonene on Iron chelating assay (39)

D-Limonene (IC_{50}= -18475.5 µM) did not show any % inhibition in iron chelating assay in comparison with Trolox (IC_{50}= 225.96 µM) as a standard.

4.6. Effect of D-Limonene on Hydroxyl radical scavenging assay (39)

D-Limonene (IC_{50}= 442.75 µM) has showed appreciable concentration dependent reduction in free radical formation by hydroxyl radical scavenging assay in comparison with Trolox (IC_{50}= 146.37 µM) as a standard.

4.7. Effect of D-Limonene on Superoxide radical scavenging assay (39; 40)

D-Limonene (IC_{50}= 692.89 µM) has showed appreciable concentration dependent reduction in free radical formation in by superoxide radical scavenging assay comparison with Trolox (IC_{50}= 105.25 µM) as a standard.

4.8. Effect of D-Limonene on lipid peroxidation (41)

The K562 (1×10^7) cells were treated with 0.5 mM, 1 mM and 2mM concentration of D-Limonene for 2 h followed by 75 µM H_2O_2 exposure for 2 h. The cells were centrifuged and washed with PBS. The pellet was resuspended in potassium chloride and homogenized. The cellular protein was precipitated and extracted followed by heating and the absorbance was measured.

D-Limonene has showed significant reduction in malondialdehyde production on K562 cells in concentration dependent manner as compared to untreated cells.

5. Estimation of *in vivo* effect of D-Limonene on subcutaneous and tail vein injection tumor xenograft model in C57BL/6 mice

5.1. Experimental animals and housing conditions

Healthy male C57BL/6 mice 4-6 week of age weighing 25-30 g were housed under standard laboratory conditions in individually ventilated cage. The mice were fed with autoclaved balanced rodent food pellet and Ampoxin (0.1 µg/ml) by drinking water, provided *ad libitum* Protocols for the experiment were approved by Institutional Animal Ethics Committee (PERD/IAEC/2017/002) prior to initiation of the experiment.

5.2. Protocol for Immunosuppression

Mice were immunocompromised by treatment with ketoconazole 10 mg/kg orally and cyclosporine 30 mg/kg intraperitoneally for 7 days. Cyclophosphamide 60 mg/kg subcutaneously was injected on days 3 and 1 before tumor cell injection (42).

Successful achievement of immunosuppression was confirmed by complete blood count. The combination of ketoconazole, cyclosporine and cyclophosphamide has significantly reduced the

white blood cells (WBC), neutrophils (NEU) and lymphocytes (LYM) in all immunocompromised animals as compared to untreated group.

5.3. Experimental design for subcutaneous injection of K562 cells in immunocompromised C57BL/6 mice

For subcutaneous injection of tumor cells, all mice were divided into 5 groups viz., normal control (n=6), disease control (n=10) and treatment group (T1, T2 and T3, (n=10 for each group)). Except normal control group, animals of all groups were immunocompromised and injected with K562 (5×10^6 cells in 0. 1 ml) cancer cell line mixed with equal volume of matrigel. The subcutaneous injection of K562 cells was given in right shoulder blade region. After the one week of tumor cell implantation treatment with D-Limonene was started.

i. **NC -** Normal control
ii. **DC -** Disease control
iii. **T1 -** D-Limonene 0.5 g/kg orally for 14 days
iv. **T2 -** D-Limonene 1.0 g/kg orally for 14 days
v. **T3** - D-Limonene 1.5 g/kg orally for 14 days

5.3.1.Evaluation Parameters

5.3.1.1. Body weight

The body weights of mice in all groups were recorded weekly throughout the study. At the end of study significant reduction in body weight was noted in disease control group as compared to normal control and D-Limonene treated group.

5.3.1.2. Evaluation of haematological and biochemical parameters

Blood was collected from retro-orbital plexus of mice in heparinized centrifuge tubes for assessment of complete blood count. The blood was also collected in clean dry centrifuge tubes, centrifuged and serum was separated for evaluation of biochemical parameters such as SGPT, SGOT and ALP. D-Limonene treatment did not show any significant change in hematological and biochemical parameters.

5.3.1.3. Effect of D-Limonene treatment on tumor growth

Tumor growth was observed at the site of injection. Tumor volume was measured externally by digital caliper using following formula:

Volume (mm^3) = (A) × (B^2)/2, where A was the largest and B the smallest diameter (mm).

D-limonene treatment at dose of 0.5 g/kg, 1.0 g/kg and 1.5 g/kg for 14 days shows dose dependent reduction in mean tumor volume as compared to vehicle treated group. However, significant reduction in tumor volume was observed only at dose of 1.0 g/kg a day and 1.5 g/kg a day with D-limonene treatment as compared to vehicle treated group.

5.3.1.4. Histopathological analysis

At the end of experiment the mice were sacrificed and tumors were excised. The tumors were stained with haematoxylin and eosin, observed under 10x and 40x magnification and photo documented by optical microscopy (IX 51; Olympus, Tokyo, Japan) equipped with a digital camera (TL4) for confirmation of presence of tumor cells.

The stained section of K562 xenograft has shown malignant cells with characteristic of large and irregular nuclei and scant cytoplasm at magnification of 100× and 400×. It also showed invasion of malignant cells in adjacent stromal tissue.

5.4. Experimental design for tail vein injection of K562 cells in immunocompromised C57BL/6 mice

For tail vein injection of tumor cells, the mice were divided into 5 groups as discussed in subcutaneous injection model. Tail vein injection of K562 cells (approx. 5 x 10^7) was given to all animals except normal control group. After an increased number of blast cells in peripheral blood treatment with D-Limonene was started.

i. **NC -** Normal control
ii. **DC -** Disease control
iii. **T1 -** D-Limonene 0.5 g/kg orally for 17 days
iv. **T2 -** D-Limonene 1.0 g/kg orally for 17 days
v. **T3** - D-Limonene 1.5 g/kg orally for 17 days

5.4.1. Evaluation parameters

5.4.1.1. Determination of model establishment by complete blood count and Giemsa differential staining of blood smear (43)

After the tail vein injection of K562 cells, blood collection from retro-orbital plexus was done weekly to confirm the development of the disease. The complete blood count was performed and the blood was analyzed for the presence of circulating blast cells by preparing the Giemsa staining of peripheral blood smear and complete blood count.

As the disease progressed, changes in haematological parameters were noted. After the 4th week of K562 cell injection, significant increase in WBC and NEU count was observed as compared to 0 week, whereas significant reduction in RBC count and Hb content was observed that confirms the establishment of the model. No significant changes in platelet count and other parameters were noted. Giemsa differential staining of peripheral blood smear showed large number of blast cells in disease control group compared with untreated group.

5.4.1.2. Body weight

Body weights of mice were checked weekly throughout the study. As the disease progressed, significant reduction in body weight was noted in disease control group as compared to normal control group. No significant change in body weight was observed in D-Limonene treatment group as compared to normal control group.

5.4.1.3. Haematological parameters

Blood was collected from retro-orbital plexus of mice and assessed for complete blood count. D-Limonene treatment at a dose of 0.5, 1.0 and 1.5 g/kg has shown significant dose dependent changes in haematological parameters as compared to disease control (DC) group. Treatment with D-Limonene at 0.5, 1.0 and 1.5 g/kg, has shown significant reduction in WBC and NEU count as compared to the DC group. The change in LYM count was non-significant with D-Limonene at 0.5 g/kg, however at doses 1.0 and 1.5 g/kg significant dose-dependent reduction was observed compared to the DC group. Changes in RBC count and Hb content were non-significant with D-Limonene treatment at 0.5 g/kg, however at doses 1.0 and 1.5 g/kg significant increase in RBC count and Hb content were observed compared to the DC group.

5.4.1.4. Biochemical parameters

The blood was collected in clean dry centrifuge tubes, centrifuged and serum was separated for evaluation of biochemical parameters such as SGPT, SGOT, ALP, lipid peroxidation, superoxide dismutase and nitrite was done at the end of study.

Significant increase in biochemical parameters such as serum SGPT, SGOT and ALP were observed in disease control group as compared to normal control group, whereas significant reduction was observed in D-Limonene treated group as compared to disease control group.

Significant dose dependent reduction in serum lipid peroxidation and nitrite level, whereas significant increase in superoxide dismutase level was observed with D-Limonene treatment as compared to disease control group.

6. Effect of D-Limonene on angiogenesis by CAM assay

Fertilized chick eggs were procured and incubated at 37ºC and 60% humidity in an egg incubator in sterile condition. On the 3rd day of incubation, an air sac was made by gently aspirating 2 ml albumin and a small window of 2 x 2 cm was made in the shell of the egg and the eggs were incubated in the incubator. On the 8th day 1 mm^3 of gelatin sponges presoaked with D-Limonene at concentration of 0, 1, 5 and 10 µg/implant (1 implant/egg, n=12 for each treatment) were implanted on the CAM of eggs and returned for incubation. On the 12th day of incubation, the eggs were observed under the microscope at 10X for counting number of the blood vessel surrounding the gelatin sponge implants.(44)

On the 4th day of drug implantation, gelatin sponge soaked with D-Limonene at concentration 1, 5 and 10 µg/implant has shown significant dose-dependent reduction in number of blood vessels on CAM as compared to implant soaked with the vehicle. Reduction in number of blood vessels on CAM was highest with D-Limonene at concentration 10 µg/implant.

7. Effect of D-Limonene on expression of VEGF protein by western blotting (45)

The K562 cells were plated in 6 well plates with D-Limonene at concentration of 0, 1, 2, 3 and 4 mM for 24 h in incubator at 37°C in a humidified atmosphere of 5% CO_2.After 24 h, the cells were washed with PBS (Phosphate buffer saline) and lysed with lysis buffer for separation of proteins. The proteins were quantified by Bradford assay and used for determination of expression of VEGF protein by western blotting.

The protein samples were denatured, loaded and electrophoresed in polyacrylamide gel (Stacking-4 % and Resolving-10%) containing running buffer. The transfer of proteins was done on PVDF membrane. The membrane was incubated overnight with primary antibody for VEGF and beta-actin (housekeeping protein) followed by blocking with 2% BSA. On next day membrane was incubated with HRP conjugated secondary antibody and developed with Luminol reagent. The blots were visualized on chemidoc system and photographed.

D-Limonene has shown significant dose dependent reduction in the expression of VEGF protein as compared to beta-actin. The inhibition of VEGF protein expression was highest at concentration of 4mM.

8. Estimation of matrix metalloproteinase (MMPs)- 2 and 9 by gelatin zymography (46)

The K562 cells were treated D-Limonene at concentration of 0, 1, 2, 3 and 4 mM and proteins were extracted and quantified in similar way discussed in protocol for western blotting. The

samples were loaded without heating and electrophoresed in polyacrylamide gel containing 1% gelatin. The gel was placed in renaturing and developing solutions respectively and stained with staining solution. Sharp clear bands of MMP-2 and 9 were visualized against dark background followed by destaining.

D-Limonene treatment has shown significant dose dependent reduction in the expression of MMP-2 and 9 proteins in gelatin zymography as compared to proteins from untreated cells. The inhibition of MMP-2 and 9 proteins were highest at concentration of 4mM.

G. Achievements with respect to objectives:

In present study, D-limonene has inhibited the *in vitro* growth of K562 cells in dose dependent manner without producing toxicity on primary hepatocytes isolated from mouse as compared to doxorubicin. D-Limonene has shown appreciable concentration dependent antioxidant activity by reducing the free radical formation in all assays except in iron chelating assay. In the *in vivo* studies on K562 tumor implanted C57BL/6 mice; D-limonene shows regression of tumor growth in dose dependent manner in subcutaneous and tail vein injection model of CML. D-Limonene has inhibited the angiogenesis in concentration-dependent manner in CAM assay. D-Limonene has also shown dose dependent inhibition in the expression of VEGF, MMP-2 and 9 proteins in K562 cells.

H. Conclusion:

In conclusion, D-Limonene inhibits CML progression through inhibition of oxidative stress and the factors involved in angiogenesis and metastasis. However, more studies are needed to elaborate the complicated signaling pathway involved in CML and to establish its role for the treatment of the disease.

I. List of papers published and Copies of the same

(A) Publications:

1. **Bhavini Shah**, Muhammad Vaseem Shaikh, Kiranj Chaudagar, Manish Nivsarkar, Anita Mehta. D-limonene possesses cytotoxicity to tumor cells but not to hepatocytes. Polish Annals of Medicine [In print]. Doi 10.29089/2017.17.00047
2. **Bhavini B. Shah**, Anita A. Mehta. In vitro evaluation of antioxidant activity of d–limonene. Asian Journal of Pharmacy and Pharmacology 2018; 4(6):883-887
3. **Bhavini B. Shah**, Ruma Baksi, Kiranj K. Chaudagar, Manish Nivsarkar, Anita A. Mehta. Anti-leukemic and anti-angiogenic effect of D–Limonene on K562 implanted C57BL/6

mice and chick chorioallantoic membrane model. Animal Models and Experimental Medicine 2018;00:1–6.

(B) Poster Presentation:

1. D-Limonene a promising anti-cancer agent: A study on K562 cells, in GUJCOST and DST Sponsored National Conference on Perspectives in Multidisciplinary Research: Changing Paradigm held at A-One Pharmacy College, SNME Campus, Anasan, Ahmedabad, Gujarat, India on 3rd March, 2018.
2. Effect of D-Limonene on viability of K562 cells: *In vitro* and *in vivo* study, in 50th Annual Conference of Indian Pharmacological Society IPSCON-2017, organized by Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM'S NMIMS, Mumbai, India on 15th - 17th February, 2018.
3. Effect of D-Limonene against K562 cells and primary hepatocytes at Recent Advancement In Pharmaceutical Development & Technology In India at A-One Pharmacy College, SNME Campus, Anasan, Ahmedabad, Gujarat, India on 4th March, 2017.

K. References:

1. Lozzio Cb Fau - Lozzio BB, BB L. 1975. - Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 45:321-34
2. George-Gay B, Parker K. 2003. Understanding the complete blood count with differential. *Journal of PeriAnesthesia Nursing* 18:96-117
3. C S, Mayerhofer M Fau - Aichberger KJ, Aichberger Kj Fau - Krauth MT, Krauth Mt Fau - Valent P, P V. 2004. - Expression of angiogenic factors in chronic myeloid leukaemia: role of the bcr/abl oncogene, biochemical mechanisms, and potential clinical implications. *Eur J Clin Invest* 2:2-11
4. WB C, GJ T. 2006. - Molecular mechanisms of human carcinogenesis. *Exs* 96:321-49
5. J F, Soerjomataram I Fau - Dikshit R, Dikshit R Fau - Eser S, Eser S Fau - Mathers C, Mathers C Fau - Rebelo M, et al. 2015. - Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136:9
6. Ganesan P, Kumar L. 2017. Chronic Myeloid Leukemia in India. *Journal of global oncology* 3:64-71
7. Bertram JS. 2000. The molecular biology of cancer. *Molecular Aspects of Medicine* 21:167-223
8. Gould MN. 1997. Cancer chemoprevention and therapy by monoterpenes. *Environmental Health Perspectives* 105:977-9
9. Rabi T, Bishayee A. 2009. Terpenoids and breast cancer chemoprevention. *Breast Cancer Research and Treatment* 115:223-39
10. Crowell PL, Gould MN. 1994. Chemoprevention and Therapy of Cancer by d-Limonene. 5:1-22
11. Klein E, Vánky F, Ben-Bassat H, Neumann H, Ralph P, et al. 1976. Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *International Journal of Cancer* 18:421-31

12. JIVRAJANI M, SHAIKH MV, SHRIVASTAVA N, NIVSARKAR M. 2014. An Improved and Versatile Immunosuppression Protocol for the Development of Tumor Xenograft in Mice. *Anticancer Research* 34:7177-83
13. Ross DM, Arthur C, Burbury K, Ko BS, Mills AK, et al. 2018. Chronic myeloid leukaemia and tyrosine kinase inhibitor therapy: assessment and management of cardiovascular risk factors. *Internal Medicine Journal* 48:5-13
14. Franklin M, Burns L, Perez S, Yerragolam D, Makenbaeva D. 2018. Incidence of type 2 diabetes mellitus and hyperlipidemia in patients prescribed dasatinib or nilotinib as first- or second-line therapy for chronic myelogenous leukemia in the US. *Current Medical Research and Opinion* 34:353-60
15. D B, Rossi Dj Fau - Weissman IL, IL W. 2006. - Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol* 169:338-46
16. Mayerhofer M, Valent P, Sperr WR, Griffin JD, Sillaber C. 2002. BCR/ABL induces expression of vascular endothelial growth factor and its transcriptional activator, hypoxia inducible factor-1α, through a pathway involving phosphoinositide 3-kinase and the mammalian target of rapamycin. *Blood* 100:3767-75
17. Winer A, Adams S, Mignatti P. 2018. Matrix Metalloproteinase Inhibitors in Cancer Therapy: Turning Past Failures Into Future Successes. *Molecular Cancer Therapeutics* 17:1147-55
18. Deryugina EI, Quigley JP. 2006. Matrix metalloproteinases and tumor metastasis. *Cancer and Metastasis Reviews* 25:9-34
19. M S, Verma S Fau - Shrikhande G, Shrikhande G Fau - Byrne CH, Byrne Ch Fau - Pride YB, Pride Yb Fau - Winkler T, et al. 2000. - The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells. *J Biol Chem* 275:24273-8
20. Sattler M, Verma S, Shrikhande G, Byrne CH, Pride YB, et al. 2000. The BCR/ABL Tyrosine Kinase Induces Production of Reactive Oxygen Species in Hematopoietic Cells. *Journal of Biological Chemistry* 275:24273-8
21. Ravi A, Alvala M, Sama V, Kalle AM, Irlapati VK, Reddy BM. 2012. Anticancer activity of Pupalia lappacea on chronic myeloid leukemia K562 cells. *DARU Journal of Pharmaceutical Sciences* 20:86
22. d'Alessio PA, Ostan R, Bisson J-F, Schulzke JD, Ursini MV, Béné MC. 2013. Oral administration of d-Limonene controls inflammation in rat colitis and displays anti-inflammatory properties as diet supplementation in humans. *Life Sciences* 92:1151-6
23. Igimi H, Watanabe D, Yamamoto F, Asakawa S, Toraishi Ki, Shimura H. 1992. A useful cholesterol solvent for medical dissolution of gallstones. *Gastroenterologia Japonica* 27:536-45
24. L Y, J Y, Z S. 2017. - D-limonene exhibits anti-inflammatory and antioxidant properties in an ulcerative colitis rat model via regulation of iNOS, COX-2, PGE2 and ERK signaling pathways. *Mol Med Rep* 15:2339-46
25. Crowell PL, Ayoubi AS, Burke YD. 1996. Antitumorigenic Effects of Limonene and Perillyl Alcohol Against Pancreatic and Breast Cancer. In *Dietary Phytochemicals in Cancer Prevention and Treatment*:131-6. Boston, MA: Springer US. Number of 131-6 pp.
26. Nakaizumi A, Baba M, Uehara H, Iishi H, Tatsuta M. d-Limonene inhibits N-nitrosobis(2-oxopropyl)amine induced hamster pancreatic carcinogenesis. *Cancer Letters* 117:99-103
27. Miller JA, Thompson PA, Hakim IA, Chow H-HS, Thomson CA. 2011. d-Limonene: a bioactive food component from citrus and evidence for a potential role in breast cancer prevention and treatment. *Oncology Reviews* 5:31-42
28. Haag JD, Lindstrom MJ, Gould MN. 1992. Limonene-induced Regression of Mammary Carcinomas. *Cancer Research* 52:4021-6
29. Lu XG, Zhan LB, Feng BA, Qu MY, Yu LH, Xie JH. 2004. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. *World J Gastroenterol* 10:2140-4

30. Klein E Fau - Ben-Bassat H, Ben-Bassat H Fau - Neumann H, Neumann H Fau - Ralph P, Ralph P Fau - Zeuthen J, Zeuthen J Fau - Polliack A, et al. 1976. - Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *Int J Cancer* 18:421-31
31. E. KJ, J. GP, E. HD, M. LM, M. KS, F. TB. 1982. Morphologic and functional studies of mouse hepatocytes in primary culture. *The Anatomical Record* 204:231-43
32. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65:55-63
33. Guguen-Guillouzo C. 2002. Isolation and Culture of Animal and Human Hepatocytes. In *Culture of Epithelial Cells*:337-79: John Wiley & Sons, Inc. Number of 337-79 pp.
34. Klaunig JE, Goldblatt PJ, Hinton DE, Lipsky MM, Chacko J, Trump BF. 1981. Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro* 17:913-25
35. Williams GM, Bermudez E, Scaramuzzino D. 1977. Rat hepatocyte primary cell cultures. *In Vitro* 13:809-17
36. Brand-Williams W, Cuvelier M, Berset C. 1995. Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie* 28:25–30
37. Thaipong K, Boonprakob U, Crosby K, Zevallos L, Byrne D. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis* 19:669 – 75
38. Benzie I, Strain J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: the FRAP assay. *Analytical Biochemistry* 239:70–6
39. Alam MN, Bristi N, Rafiquzzaman M. 2013. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal* 21:143–52
40. Meyer A, Isaksen A. 1995. Application of enzymes as food antioxidants. *Trends Food Sci. Technol* 6:300–4
41. Ge H, Kong X, Shi L, Hou L, Liu Z, Li P. 2009. Gamma-linolenic acid induces apoptosis and lipid peroxidation in human chronic myelogenous leukemia K562 cells. *Cell Biology International* 33:402-10
42. M J, MV S, N S, M N. 2014. - An improved and versatile immunosuppression protocol for the development of tumor xenograft in mice. *Anticancer Res* 34:7177-83
43. Wang L-Q, Wang R, Li X-X, Yu X-N, Chen X-L, Li H. 2015. The anti-leukemic effect of carnosic acid combined with adriamycin in a K562/A02/SCID leukemia mouse model. *International Journal of Clinical and Experimental Medicine* 8:11708-17
44. Steiner R. 1992. Angiostatic activity of anticancer agents in the chick embryo chorioallantoic membrane (CHE-CAM) assay. In *Angiogenesis: Key Principles — Science — Technology — Medicine*, ed. R Steiner, PB Weisz, R Langer:449-54. Basel: Birkhäuser Basel. Number of 449-54 pp.
45. Mahmood T, Yang P-C. 2012. Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences* 4:429-34
46. Toth M, Sohail A, Fridman R. 2012. Assessment of Gelatinases (MMP-2 and MMP-9) by Gelatin Zymography. In *Metastasis Research Protocols*, ed. M Dwek, SA Brooks, U Schumacher:121-35. Totowa, NJ: Humana Press. Number of 121-35 pp.